Platelets

Covert Regulators of Lymphatic Development

Cara C. Bertozzi, Paul R. Hess, Mark L. Kahn

Abstract—The field of platelet biology has rapidly expanded beyond the classical role of platelets in preventing blood loss and orchestrating clot formation. Despite the lack of transcriptional ability of these anuclear cell fragments, platelet function is now thought to encompass such diverse contexts as tissue repair, immune activation, primary tumor formation, and metastasis. Recent studies from multiple groups have turned the spotlight on an exciting new role for platelets in the formation of lymphatic vessels during embryonic development. Genetic experiments demonstrate that Podoplanin, a transmembrane protein expressed on lymphatic endothelial cells, engages the platelet C-type lectin-like receptor 2 (CLEC-2) when exposed to blood, leading to SYK-SLP-76-dependent platelet activation. When components of this pathway are disrupted, aberrant vascular connections form, resulting in blood-lymphatic mixing. Furthermore, platelet-null embryos manifest identical blood-lymphatic mixing. The identification of platelets as the critical cell type mediating blood-lymphatic vascular separation raises new questions in our understanding of lymphatic development and platelet biology. (Arterioscler Thromb Vasc Biol. 2010;30:2368-2371.)

Key Words: platelets  vascular biology  lymphatics

Hematopoiesis and vascular development have shared origins, as progenitors of both lineages initially arise in extraembryonic blood islands. In the embryo, endothelial precursors surround clusters of primitive hematopoietic precursors before vascular lumen formation. Definitive hematopoietic stem cells subsequently arise from a subset of competent endothelial cells of the dorsal aorta in the aorta-gonad-mesonephros region of the developing embryo. There has been a long and heated debate over the contribution of bone marrow–derived cells to endothelium during development that has been complicated by the close spatiotemporal association and common molecular markers between the 2 lineages. Fatemapping studies conducted in blood vessels using Vav-Cre transgenic mice showed that most, if not all, endothelial cells are not of hematopoietic origin. However, the distinct blood-lymphatic mixing vascular phenotype in mice lacking the hematopoietic proteins SYK or SLP-76 suggested that there could be a small but critical requirement for bone marrow–derived endothelium. This review will highlight definitive evidence that SYK/SLP-76 knockout vascular abnormalities do not arise from a failure of bone marrow–derived cells to incorporate into the vasculature but rather from the requirement of hematopoietic cells, specifically platelets, to regulate blood-lymphatic separation. We present contributions by a number of groups that describe the discovery of a novel embryonic role for platelets in regulating vascular development.

Lymphatic Development

Lymphatic vessels form an extensive vascular network that facilitates immune trafficking and surveillance, maintains tissue fluid homeostasis, and absorbs dietary lipids in the intestine through mesenteric lacteals. These specialized vessels are composed of lymphatic endothelial cells (LECs) that originate in the cardinal vein as venous endothelial cells. Starting at embryonic day (E) 9.75, polarized induction of PROX1, a homeobox transcription factor, is necessary and sufficient to activate the lymphatic gene profile. These LECs then migrate away from the cardinal vein, largely in response to vascular endothelial growth factor receptor-3/Neuropilin-2 signaling induced by vascular endothelial growth factor-C in the mesoderm. The LECs assemble into vessels to form a de novo collecting system, parallel to but separate from the blood vessels, sprouting centrifugally from the initial sacs and migrating deeper into tissues to form the inner lymphatic plexus. The resulting collecting vessels form luminal valves to prevent backflow and are covered by mural cells, whereas the blind-ended capillaries lack mural cells and remain largely uninvested by pericytes. Lymphatic capillary endothelium is characterized by loose intercellular junctions that facilitate the homeostatic function of these vessels.

A careful kinetic study of murine intestinal lymphatic development reveals that mature blood vessels are established days before lymphatics invade the mesentery and the intestinal tube. By E13.5 to E14.5, although local blood vessel
development is complete, there are few or no lymphatics in
the wall of the small intestine, and immature lymphatics have
just begun to invade the mesentery. By E16.5, there has been
a remarkable increase in branching and further migration into
the intestinal tube, but it is not until E19.5 that they have
formed a mature lymphatic network capable of transporting
the large amounts of fat required for nutritional absorption.9
The completed vascular and lymphatic endothelial networks
connect only at the thoracic duct in the mouse, where the
collected lymphatic fluid is returned to the blood circulation
through the subclavian vein.5,10,11

Blood-Lymphatic Mixing
Mice lacking SYK,12,13 SLP-76,14 or PLCγ-215 develop
blood-lymphatic mixing in distinct vascular beds at specific
time points during embryonic development. This is the result
of primary misconnections between lymphatics and blood
vessels.14 Disruptions in normal development are first detect-
able at E11.5 as the nascent lymphatic sacs are emerging from
the cardinal vein along the antero-posterior axis of the
embryo. Small connections between lymphatic and venous
endothelial cells persist along the cardinal vein in the mutant
mouse, resulting in downstream filling of the superficial dermal
lymphatics with blood and general edema by midgestation.
Lymphatic development continues largely unimpeded, but
severe vascular abnormalities also become apparent in the
intestine by E16.5. Mesenteric lymphatics are invading the
vascularized intestinal tube, and the mutants develop a gross
perturbation of what are normally separate, arborized vessels.
The mesenteric lymphatic vessels themselves appear struct-
urally normal, but once these vessels reach the intestinal wall
they develop myriad connections with the blood circulation.
Real-time injection of fluorescent dextran into the circulation
reveals that the intestinal lymphatics become perfused with
dextran and are functionally indistinguishable from their
venous counterparts. The majority of these animals die
perinatally.14 Notably, some vascular beds in these animals
appear completely unaffected, including those found in the
lungs, where there is a high density of both lymphatic and
blood vessels.

Hematopoietic Requirement During
Lymphatic Development
The observation that SLP-76 knockout mice develop a
primary vascular defect was particularly surprising because
expression of this protein is confined to the hematopoietic
compartment, where it nucleates signaling complexes down-
stream of immunoreceptor activation. Expression of SLP-76
has not been detected in endothelial cells, even at the cardinal
vein during the emergence of LECs.14 Reconstitution of the
bone marrow and circulating blood cells in lethally irradiated
wild-type mice with Slp-76−/− hematopoietic cells results in
the development of intestinal blood-lymphatic mixing, high-
lighting the requirement for SLP-76 in bone marrow–derived
cells.14 Furthermore, transgenic rescue of SLP-76 in a limited
profile of hematopoietic cells but not in endothelial cells is
sufficient for normal lymphatic development.16 Runx1−/− mice,
which fail to undergo definitive hematopoiesis, demon-
strate similar blood-filled lymphatic sacs at E11.5, also
suggesting a hematopoietic requirement for normal lymphatic
development.5 Careful analysis of hematopoietic lineage
tracing using a Runx1-MER-Cre-MER inducible knock-in
mouse17 to examine the emerging lymphatic endothelial
sacs failed to reveal any direct contribution of bone marrow–
derived cells to these vessels.5 Finally, although hematopoietic-specific deletion of SLP-76 using Vav-Cre
confers the vascular phenotypes seen in the straight knockout,
lineage tracing in these mice also failed to demonstrate any
hematopoietic origin of LECs, even in the highly affected gut,
where the requirement is greatest.18

Loss of Podoplanin on LECs Leads to
Blood-Lymphatic Mixing
How does a hematopoietic signaling pathway regulate lymph-
atic vessel growth and development? A major insight into
this question came with the discovery that Podoplanin
(PDPN)-null mice exhibit blood-lymphatic mixing and vas-
cular abnormalities that closely phenocopy those of mice
lacking SYK, SLP-76, and PLCγ2.18–20 PDPN is a heavily
O-glycosylated type I transmembrane protein whose expres-
sion is largely restricted to glomerular podocytes, lung
alveolar type I cells, and LECs but is not seen in hematopoi-
etic cells or blood endothelial cells. Endothelial loss of the
T-synthase enzyme required for PDPN synthesis is sufficient
for blood-lymphatic mixing like that observed in mice lack-
ing the immune receptor signaling pathway in blood cells.19
Radiation chimeras confirm that the requirement for PDPN is
not in blood cells,18 consistent with an essential role in LECs.
These studies and the finding that loss of PDPN magnified
the penetrance of the vascular mixing phenotype in Vav-Cre;
Slp-76fl/fl animals placed PDPN in the same pathway as
SYK, SLP-76, and PLCγ2, but in a different cell type.

PDPN and SYK-SLP-76 Signaling Are Linked
by CLEC-2 receptors on Platelets
PDPN, also known as aggrus, was identified as a tumor cell
surface protein capable of initiating platelet aggregation.21,22
The mechanism for this response was recently identified as
PDPN-mediated activation of a novel platelet receptor, C-
type lectin-like receptor 2 (CLEC-2), that is highly ex-
pressed on platelets.23 The intracellular tail of CLEC-2
contains a single YxxL motif that initiates downstream
signaling through SYK and SLP-76 on ligand engagement
of a CLEC-2 dimer,24 providing a molecular explanation for
how PDPN and these hematopoietic signaling proteins may
be linked. Genetic deletion of CLEC-2 in mice reveals a
striking phenocopy of the blood-lymphatic mixing defects
seen in mice deficient for SLP-76 or PDPN.18,25 Radiation
chimeras reconstituted with Clec-2−/− fetal liver cells de-
velop the intestinal mixing phenotype, confirming a hematopo-
ietic requirement for CLEC-2.19 In situ and flow cytometry
studies indicate that CLEC-2 is restricted to platelet-
generating megakaryocytes and platelets in the developing
embryo,18 although in older animals it is also reported to be
present on peripheral blood neutrophils.26 These studies
suggested that PDPN on LECs activates CLEC-2 receptors
and downstream SYK-SLP-76 signaling in platelets to initiate
and maintain blood-lymphatic vascular separation. This un-
Platelets regulate blood-lymphatic vascular separation. A, Lymphatic vascular development begins at the cardinal vein in the embryo, where venous endothelial cells give rise to the first LECs to form the lymph sacs. Platelet microthrombi are present on LECs at the cardinal vein in wild-type embryos but are absent in Clec-2−/−, Slp-76−/−, and Pdpn−/− embryos, which develop blood-filled lymph sacs. These primary misconnections lead to downstream blood-filled lymphatics. B, Abnormal blood-lymphatic vascular connections form in the intestines of Clec-2−/−, Slp-76−/−, and Pdpn−/− mice. A proposed mechanism of intestinal blood-lymphatic mixing is shown, where vascular misconnections arise because of angiogenic invasion between blood and lymphatic vasculature. C, PDPN on the surface of lymphatic endothelium binds to platelets through the CLEC-2 receptor, leading to SLP-76-dependent platelet activation. Platelet activation mediates vascular separation through an unknown mechanism.

Future Directions

It remains unknown how abnormal blood-lymphatic vascular connections arise in the absence of CLEC-2-PDPN signaling. Platelet-LEC interactions can be seen in the cardinal vein during the specification of PDPN-expressing LECs from venous endothelial cells, but similar interactions have not been detected in the intestine, where large numbers of blood-lymphatic connections are formed. Because both PDPN and CLEC-2 are transmembrane proteins, the mechanism requires direct contact between LECs and platelets. The use of platelets as a means of sensing blood vessel contact by LECs makes sense, as platelets are one of the few blood cell types that cannot extravasate and enter lymphatic vessels, even following trauma. Precisely how platelet activation by LECs negatively regulates the formation of LEC connections to blood vessels remains speculative. The requirements for SYK and SLP-76 indicate a need for platelet activation downstream of the PDPN-CLEC-2 interaction. The fact that mice lacking platelet integrins required for platelet aggregation do not form blood-lymphatic connections suggests that the formation of a platelet plug, the hallmark of platelet-mediated hemostasis, may not be the key step downstream of platelet activation. Instead, it is tempting to speculate that platelet degranulation may release regulators of LEC growth that inhibit the formation of blood-lymphatic connections. Platelet α-granules are known to contain numerous angiogenic regulators, supporting the hypothesis that degranulation is the mechanism by which platelet activation controls lymphatic growth. Additional genetic studies will be required to test the effects of such agents on LEC growth and lymphatic development.

The studies described above are also significant because they describe a clear embryonic role for platelets that is not connected to hemostasis. Platelet activation has been associated with many nonhemostatic roles in mature animals, including inflammation, wound healing, primary tumor growth, and tumor metastasis, but few embryonic roles have been defined. The lack of an embryonic phenotype in NF-E2-deficient mice lacking most but not all platelets has suggested that platelets do not have such roles, but this is clearly not the case, and it seems likely that future studies will identify other roles for platelets in regulating development of the cardiovascular and other systems.

Finally, it remains unclear whether PDPN or CLEC-2 play roles outside lymphatic vascular development. The number of CLEC-2 receptors on the surface of human or mouse platelets has not yet been determined. However, comparison of hematopoietic and megakaryocyte gene expression libraries, as well as in situ hybridization studies of mouse megakaryocytes and binding of anti-CLEC-2 antibodies and PDPN-Fc fusion proteins, suggests that CLEC-2 may be one of the most highly expressed activating receptors on the platelet surface. In vitro studies of Clec-2−/− platelets have suggested that CLEC-2 deficiency does not affect collagen-induced aggregate formation. Furthermore, tyrosines in the YxxL motif of platelet CLEC-2 receptors are not phosphorylated on flow over collagen, suggesting that CLEC-2 is not involved in platelet activation by classical hemostatic stimuli. The exis-
tence of a PDPN-independent role for CLEC-2 remains unclear and is an open question in the field. Future studies addressing the hemostatic role(s) of CLEC-2 and PDPN are likely to yield new insights into platelet biology.

Disclosures

None.

References

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